

***SRY*-Positive 46, XY male with vanishing testis syndrome, feminization and gynecomastia**

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ABSTRACT

The vanishing testis with maleness is a rare syndrome with frequency of 1 in 20,000 males. Here, we report about a 30 years old male subject with vanishing testis syndrome, feminization and gynecomastia. Follicle stimulating hormone (FSH) and Leutinizing hormone (LH) levels were elevated whereas testosterone was below normal and anti-mullerian-hormone level was undetectable in the patient. The chromosomal analysis and DNA analysis of *SRY* and *ZFY*, *DAX-I*, *AZFa*, *AZFb*, *AZFc* and heterochromatic region of Y chromosome with STS primer (sY160) were done to detect any genetic changes at specified sites (both at chromosomal and molecular level). Karyotyping confirmed patient as 46, XY male, with no evidence of mosaicism in blood cells. PCR amplification of *SRY* gene indicated that the *SRY* gene of the patient was normal. PCR amplification of *SRY*, *ZFY*, *DAX-I*, *AZFa*, *AZFb*, *AZFc* gene and Y chromosome heterochromatic region using STS primer sY(160) did not reveal any microdeletions. The anti-mullerian-hormone level was undetectable indicating that the patient didn't have any testicular tissue in scrotum. Increased levels of FSH, LH and reversed androgen: estrogen ratio might have given rise to gynecomastia in the patient. *SRY*-positive 46,XY male with vanishing testis might be due to torsion of testis during descent in fetal period. The torsion of testis might have caused vascular occlusion and thereby regression of testicular tissue occurred, but the exact genetic condition yet to understand.

Keywords: Gynecomastia, *SRY*, *ZFY*, *DAX-I* gene, testis vanishing syndrome.

INTRODUCTION

The vanishing testis syndrome is defined as embryonic testicular regression sequence in genetic and phenotypic males.^{1,2} Such individuals are genetically male 46,XY with absence of recognizable testis structure and absence of mullerian duct system.³ Vanishing testis syndrome affects one in 20,000 males.⁴ Some patients are reported with ambiguous external genitalia or micropenis, but most of the individuals have a normal male phenotype.⁵ The male sex differentiation and development of the penis and scrotum is dependent on the production of anti-mullerian-hormone (AMH) and androgens.⁶ Although, the testis might have formed after this initial hormonal activity, in stage of late foetal or early neonatal life, due to some unknown reason testis degenerated, which leads to the condition called testis vanishing syndrome or bilateral anorchia.^{7,8}

Development of the male gonads is a very interesting and balanced play of the genetic, endocrinal and vascular systems. Testes are determined by the presence of the *SRY* gene located on Y chromosome. The downstream regulation pathway depends on the appropriate functioning of the *SRY* and its candidate genes for successful and complete development of male gonads.⁹

The *DAX-I* (dosage sensitive sex-reversal, congenital adrenal hypoplasia etc, on the X chromosome gene I) alternatively called *NROB1* (nuclear receptor subfamily O, group B, member 1) is expressed in tissues involved in steroid hormone production, reproductive function as well as testis development.^{10,11} Another *ZFY* (Zinc-finger-Y) gene is a candidate on the human Y chromosome and is postulated to initiate testis differentiation during embryogenesis.¹² *ZFY* gene sequences are present in most sex-reversed XX males and absent in some XY females. Hence, the *ZFY* gene is directly related to testis formation and male phenotype development in human.¹³ These observations suggested that, a molecular change in *DAX-I* or *ZFY* gene is one of the reasons for gonadal defects in male.^{11,13} Here, we confirmed the presence of *SRY* on Y chromosome and presenting molecular analysis of *ZFY*, *DAX-I*, *AZFa*, *AZFb*, *AZFc* gene. But there was no known reason that explain why the testis vanish or doesn't develop. It could be either genetic, environmental or a vascular events.

MATERIALS AND METHODS

Case history and clinical details: A 30 yrs old male reported at surgery OPD with a complaint of gynecomastia. After physical examination of patient, it

Table-1: Polymerase Chain Reaction programme and Primer sequences used in this study.

Gene	Primer symbol	Primer sequence(5'-3')	PCR program	Product size (bp)
SRY	SRY1	GAATATTCCTGCTCTCCGGA GCTGGTGTCCATTCTTGAG	94°C- 12 min (94°C-1 min, 65°C-1 min, 72°C-2 min) x 35 cycles, 72° C- 10 min	472
	SRY2	CCCGAATTCGACAATGCAATCATAT GCTTCTGCCTGTAGCGGTCCCGT TGCTGCGGTG	94°C- 12 min (94°C-1 min, 58°C-1 min, 72°C-2 min) x 35 cycles, 72 °C- 10 min	609
	SRY3	GACAGCAGTAGAGCAGTCAGGGAG GCTGTAGCGGTCCCGTTGCTGCG	94°C- 12 min (94°C-1 min, 65°C-1 min, 72°C-2 min) x 35 cycles, 72 °C- 10 min	868
ZFY	ZFY 1	GGTCTGCAGACTCTTCTAACTTCT TGCAGTACTCACACCG	94°C- 12 min (94°C-45 s, 60°C-45 s, 72°C-1 min) x 30 cycles, 72°C- 10 min	603
-I DAX	DAX1	CCGCGCCCTTGCCAGACCGC CGCCTGCGCTTGATTTGT	94°C- 12 min (94°C-1 min, 58°C-45 s, 72°C-1.5 min) x 30 cycles, 72°C- 10 min	786
	DAX2	CGCGCAGAGGCCAGGGGGTA AAGCCCCGACACTCTCCTGATCACTG	94°C- 12 min (94°C-45 s, 58°C-45 s, 72°C-2 min) x 30 cycles, 72°C- 10 min	750
	DAX3	TTGGGTCTTGTTTAATTGGGATGAAC CATGAAATTGCTACACTTGTGAAAA	94°C- 12 min (94°C-1 min, 58°C-1 min, 72°C-1.5 min) x 30 cycles, 72°C- 10 min	644

was found that (i) both scrotal sacs were empty, (ii) gynecomastia were present (iii) the penis was normal looking, (iv) stature of body and pubic hair pattern was feminine, (v) length of the lower body segment was distinctly longer than upper segment. Suspecting a patient of Klinefelter's syndrome, he was referred to our institutional cytogenetic lab for karyotyping.

CLINICAL INVESTIGATIONS:

- 1) Radiological:** On ultrasonography revealed bilateral anorchia. Prostate and seminal vesicle were reported to be normal, vas deference were atrophied. While rests of the abdominal and pelvic organs were normal.
- 2) Hormonal:** Plasma hormone concentrations were measured by Radio-Immuno-assay method (RIA).

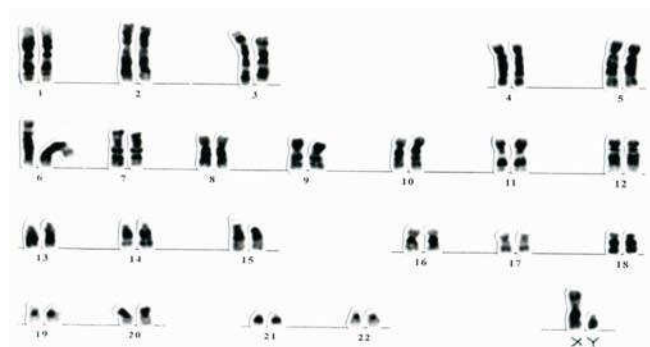


Fig 1: Karyotype: 46, XY.

Serum concentrations of Leutinizing hormone(LH) and Follicle stimulating hormone (FSH) were elevated at 41.15 mIU/ml (normal 3.1-34.6 mIU/ml) and 85.54mIU/ml (normal 1.4- 18.1 mIU/ml), respectively. Testosterone hormones level was reduced to 7.76 ng/dl (normal adult level, 241-827 ng/dl). Estrogen (E2) level was also decreased up-to 5pg/ml (normal values 7.63-42.6 pg/ml). Anti mullerian hormone concentration was undetectable (normal value 3.0-5.4 ng/ml).

- 3) Semen analysis:** Semen analysis¹⁴ showed that the subject had aspermia.
- 4) Cytogenetic analysis:** Karyotyping was done following lymphocyte culture.¹⁵ Total of 50 G-banded metaphases were analysed to detect any heterozygosity among the cell populations. The patient had normal male karyotype i.e. 46,XY (Fig. 1).
- 5) DNA Isolation and PCR analysis:** DNA was extracted from peripheral blood lymphocytes of the patient. Simultaneously lymphocytes were collected from normal fertile male and a normal fertile female using the protocol described in earlier studies¹⁶. DNA samples were quantified spectrophotometrically by measuring the absorbance at 260 nm. Serial dilutions of DNA were made up to 50 ng/µl concentration. We selected genes residing in different region of Y

chromosome to look at the presence or absence of Y chromosome derived sequences. *SRY* gene was amplified (Applied Biosystem, California, USA) with three pairs of primers covering 478bp, 600bp, 868bp around HMG box of the gene. Similarly, *ZFY* gene was amplified with a pair of primer covering one of the zinc-finger regions. PCR primers (Merck, Bioscience, Bangalore) were used for *SRY*, *ZFY*, and *DAX-I* genes^{16,17}(Table-1). Full coding regions of *DAX-I* gene along with exon-intron junctions were amplified. Spermatogenic genes *AZFa* (sY84, sY86), *AZFb* (sY127, sY134), *AZFc* (sY254, sY255) and a part of heterochromatic region of Y chromosome (sY160) were amplified by PCR to look for presence/absence of these genes. Specific STS primers were taken for analysis of the spermatogenic genes. For each PCR amplification, 50 µl reaction mixtures were used. PCR reactions consisted of 5.0 µl of PCR reaction buffer (5X), 3µl of MgCl₂ (25mM), 2.5 µl of dNTPs (10mM), 10 pM of each primer, 2.0 unit of Taq DNA Polymerase(GeNei™, Bangalore), 50 ng of DNA template and 37 µl sterile distilled water. All the amplifications were performed in three independent PCRs each time with positive and negative controls. Reaction products were stored at 4°C until they were loaded onto agarose gels for analysis. The PCR reaction products were separated on 1.8% agarose gels by electrophoresis in Tris-boric acid-ethylenediamine tetraacetate buffer at room temperature using a 50-60 voltage for 1hrs.

RESULTS

Cytogenetic analysis: Karyotype of the patient showed 46,XY chromosome complement (Fig. 1). Analysis of 50 metaphases showed no evidence of mosaicism and structural or numerical chromosomal abnormality.

PCR Analysis: Successful amplification and the Molecular analysis of X chromosome marker *DAX-I* gene and Y chromosome specific markers *SRY*, *ZFY*, *AZFa*, *AZFb*, *AZFc* and heterochromatic region revealed their presence in patient's DNA and no microdeletions were observed at the specific site of the above mentioned genes(Fig. 2). Possibility of PCR contamination was excluded by sterile distilled water to use as a negative control. All the above-mentioned markers showed consistent amplification in positive control as a normal fertile male and no amplification in the negative control.

DISCUSSIONS

Vanishing testis syndrome or anorchia is a very uncommon type of testicular abnormality.¹⁸ As testicular function becomes impaired, testicular secretion of both the testosterone and estrogen decreases¹⁹ and blood

serum AMH value also decreased up-to undetectable levels. These results indicated absences of testicular tissue.⁶ In our patient the concentration of serum FSH and LH were highly increased whereas serum testosterone and estrogen level decreased below the normal range. Lower testosterone level with intact hypothalamus causes marked rise in LH and FSH levels above the normal by stimulating the pituitary gland with negative feed back mechanism.^{20,21} Estrogen in a male with anorchia is almost exclusively derived from extra glandular acromatization of adrenal androgens. At this point estrogen formation, although low, is relatively higher than that of the testosterone and thus reversing the androgen: estrogen ratio, causing gynecomastia in male.¹⁹ In this clinical report also we have observed the increased levels of FSH and LH, reversed androgen: estrogen ratio, because of which the gynecomastia might have developed in the subject.

The testis develops in the abdomen from the genital ridge at the 8th week of the gestational age, under influence of *SRY* gene on Y chromosome.^{3,22} We have confirmed that the testis determining gene, *SRY*, was not mutated in the subject (Fig. 2). Assuming that testis vanishing syndrome was the result of clinical spectrum of 46, XY gonadal dysgenesis, our interest was to study other sex determining genes, which act downstream regulation of *SRY* such as *ZFY* and *DAX-I*.¹⁷ X-linked locus DSS (dosage sensitive sex reversal) containing orphan nuclear receptor, *DAX-I* gene at Xp21 region reported to function as an early mediator of testes development.¹⁰ The mutation of *DAX-I* gene causes the hypogonadism. *ZFY* gene on Y chromosome plays an important role in the differentiation of the indifferent gonads to testis during

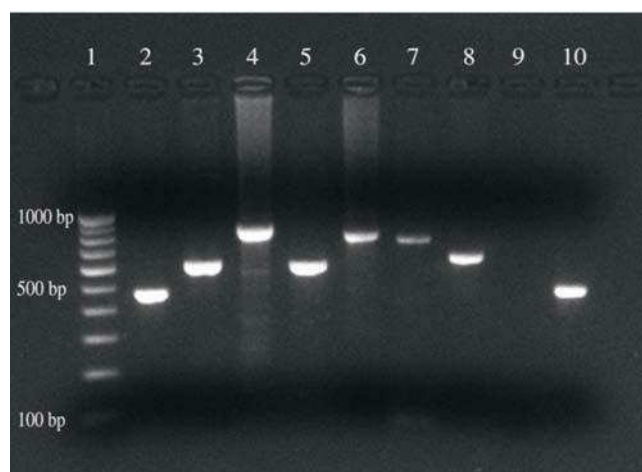


Fig. 2: Gel electrophoresis of the PCR amplicons of the *SRY*, *ZFY* and *DAX-1* genes. Lane 1: 100bp ladder. Lane 2-4: *SRY*1 (472bp), *SRY*2 (609bp), *SRY*3 (868bp) amplification respectively. Lane 5: *ZFY* (603bp). Lane 6-8: *DAX-1*(786bp), *DAX-2*(750bp), *DAX-3*(644bp) amplification respectively. Lane 9 & 10: *SRY* set of primers for normal fertile female and normal fertile male *SRY*1(472bp) respectively.

embryogenesis. The *ZFY* gene is responsible for testis determination, the testis determining factor has also important factor of this male gonad development events.¹³ Insulin like 3 (*INSL3*) and Leucine rich repeat containing G protein coupled receptor 8 (*LGR8*) gene on the Y chromosome regulate the descent of testis. If the testis fails to reach in the scrotum, it is called Cryptorchidism. Mutation has been reported in *INSL3* and *LGR8* genes in a few of the patients with cryptorchidism.²³

The anti mullerian hormone level was undetectable and increasing level of FSH and LH indicates that the patient didn't have any testicular tissue in scrotum.²⁰ Somatic changes like abnormal body stature, female hair pattern and gynecomastia are due to the disturbed hormonal profile because of agonadal condition.²⁴ The patient had well developed external and internal genitalia and there was no remnant of the mullerian duct derivatives. The explanation to this anomalous situation was possibly due to normal functioning of testes with secretion of AMH and testosterone but disappeared after 14th week.

This may be due to unknown genetics / vascular disturbance in early fetal life. Some authors^{25,26} had explained this may be due to unknown nutrition in *INSL3* and *LGR8* genes causing torsion and abnormal descent of testes.

The torsion of testis during descent causes vascular occlusion which might have initiated to vanish the testis. There was no known genetic reason explaining the etiology of vanishing testis syndrome but it needs more genetic exploration to reveal the etiology of such rare syndrome.

ACKNOWLEDGEMENTS

We are very thankful to Indian Council of Medical Research, New Delhi, India for financial support. All the authors are greatly thankful for invaluable appreciating technical support of Mr. V. P. Kavinesan and Mrs. C. M. Chuhan.

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